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# Extracellular Enzyme Activity and Microbial Diversity Measured on Seafloor Exposed Basalts from Loihi Seamount Indicate the Importance of Basalts to Global Biogeochemical Cycling

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**Seafloor basalts are widely distributed and host diverse prokaryotic communities, but no data exist concerning the metabolic rates of the resident microbial communities. We present here potential extracellular enzyme activities of leucine aminopeptidase (LAP) and alkaline phosphatase (AP) measured on basalt samples from different locations on Loihi Seamount, HI, coupled with analysis of prokaryotic biomass and pyrosequencing of the bacterial 16S rRNA gene. The community maximum potential enzyme activity ($V_{max}$) of LAP ranged from 0.47 to 0.90 nmol (g rock)$^{-1}$ h$^{-1}$; the $V_{max}$ for AP was 28 to 60 nmol (g rock)$^{-1}$ h$^{-1}$. The $K_m$ of LAP ranged from 26 to 33 μM, while the $K_m$ for AP was 2 to 7 μM. Bacterial communities on Loihi basalts were comprised primarily of *Alpha-*, *Delta-*, and*Gammaproteobacteria*, *Bacteroidetes*, and *Planctomycetes*. The putative ability to produce LAP is evenly distributed across the most commonly detected bacterial orders, but the ability to produce AP is likely dominated by bacteria in the orders *Xanthomonadales*, *Flavobacteriales*, and *Planctomycetales*. The enzyme activities on Loihi basalts were compared to those of other marine environments that have been studied and were found to be similar in magnitude to those from continental shelf sediments and orders of magnitude higher than any measured in the water column, demonstrating that the potential for exposed basalts to transform organic matter is substantial. We propose that microbial communities on basaltic rock play a significant, quantifiable role in benthic biogeochemical processes.**

Exposed seafloor basalts comprise a 600,000-km$^2$ continuous undersea habitat (1). Endolithic basalt microbes are diverse and abundant (2), and several clades of bacteria appear more likely to be encountered on basalts than elsewhere (3). Bacterial phyla display trends in abundance that reflect rock geochemistry, indicating a strong selection of microbial communities by rock composition (4). Genes diagnostic for methanogenesis, nitrogen fixation, anaerobic ammonium oxidation, denitrification, Fe reduction, and dissimilatory sulfate reduction are present in basalt microbial communities (3), indicating the potential for diverse biogeochemical transformations on basalts. However, no data are currently available for metabolic activity rates of basaltic microbes.

The relationship between the presence of prokaryotes and the activity and function of enzymes in extreme environments in general and basalts in particular is underexplored. Hydrolytic extracellular enzymes have been shown to be indicators of metabolically active bacteria, and existing data sets from various marine environments can be used for comparison with information from newly explored areas (5, 6). Additionally, in deep sea environments, organic matter is more refractory in nature, and therefore, extracellular enzyme hydrolases should play an important role in the initiation of organic matter recycling. Indeed, recent work showed that the most abundant group of *Archaea* in marine sediments produces unique extracellular enzymes for the degradation of proteins (7).

The objectives of this study were to determine to what degree microbial communities within basalts are metabolically active and to evaluate possible linkages between biomass, phylogeny, and extracellular enzyme activity and function on seafloor exposed basalts. We sampled three basalts from Loihi Seamount, located off the southeast corner of the big island of Hawaii (8), and measured the leucine aminopeptidase (LAP) and alkaline phosphatase (AP) activities in conjunction with quantitative PCR (qPCR) of 16S rRNA and pyrosequencing of the V6 region of bacterial 16S rRNA to quantify the microbial metabolism and describe the prokaryotic diversity in the collected samples. AP catalyzes the degradation of phosphate esters, organic phosphorus compounds with a C-O-P bond, supplying phosphate from phosphate esters and/or carbon products necessary for cell maintenance and growth (9–12). LAP cleaves N-terminal leucine residues from proteins and peptides and is viewed as a cell maintenance enzyme with a critical role in peptide turnover (6). The two extracellular enzymes chosen for this study were selected because their common use in ecological studies provides a wealth of prior data to allow comparison of community enzyme activities from this study to those in other areas.

## MATERIALS AND METHODS

**Rock collection and sampling.** Three basalt rocks were collected in October 2009 at Loihi Seamount, 20 miles southeast of the coast of the big island of Hawaii, from the research vessel R/V *Kilo Moana* using the remotely operated underwater vehicle (ROV) *Jason II* (Fig. 1). Basalt J2477 was collected from the Ula Nui site (13) at a depth of 4,987 m. This sample was very glassy, with ~3-mm olivine phenocrysts, and was vesicular, fri-

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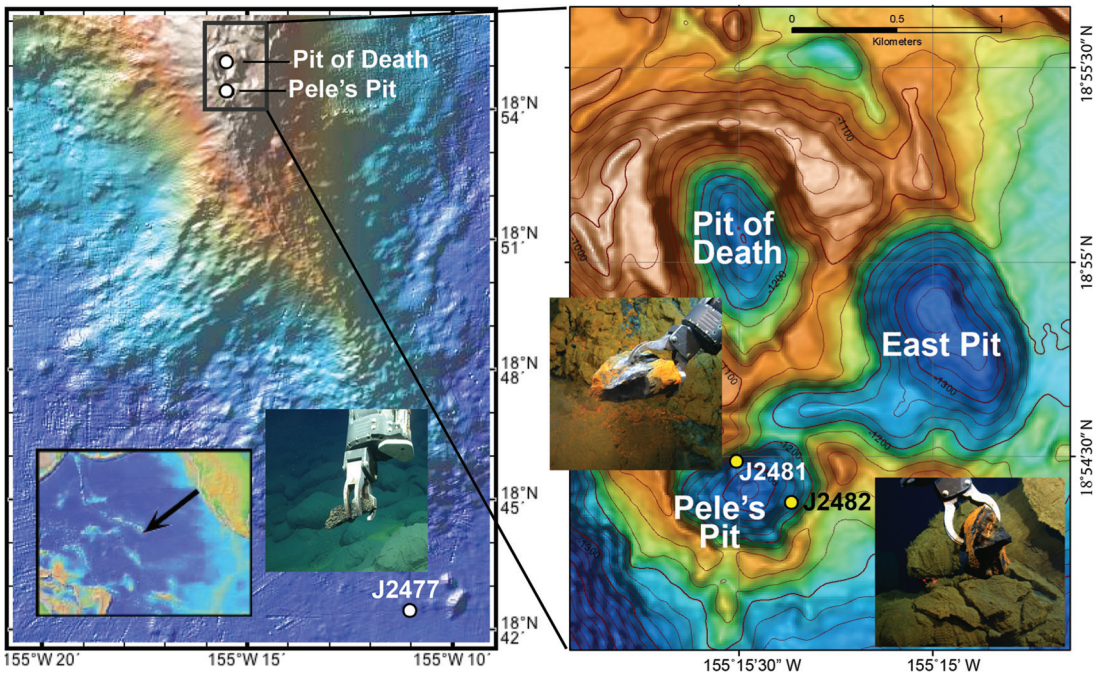


**FIG 1** Loihi Seamount, with station locations indicated. The inset at the bottom left of the left panel indicates the location of Loihi in the Pacific Ocean, and the rectangle at the top indicates the location of the area highlighted in the right panel. Photographs in both panels are of the samples at the time of collection by ROV *Jason II*. The left panel was made with GeoMapApp (www.geomapapp.org).

able, and displayed visible oxide staining. Basalt J2481 was an intact pillow with an altered rind collected in cold water (not near diffuse venting) from Marker 2 at the Lohiau site (14) at a depth of 1,178 m. Basalt J2482 was an intact pillow with an altered rind collected from a talus field in the southeast corner of Pele's Pit at a depth of 1,198 m. All samples were placed in bioboxes aboard the ROV that were sealed with the sample inside for the remainder of the dive to prevent interaction with the water column away from the sample collection site. Once the ROV was retrieved and on board the research vessel, the samples were processed using sterile chisels in a sterilized stainless steel container. Harvested rock chips for use in enzyme assays were stored until analysis in a sterile glass jar filled with Sigma sterile seawater (Sigma-Aldrich, St. Louis, MO). Rock chips for use in analysis of microbial composition were immediately placed at −80°C.

**Nutrient water sampling and analysis.** Background water samples were collected away from venting sites using Niskin bottles attached to the side of ROV *Jason II*. After vehicle recovery, the samples were filtered through a 0.2-μm polycarbonate filter and frozen at −20°C until analysis. $NO_2$ plus $NO_3$ ($NO_x$) was measured using the chemiluminescence method with a $NO_x$ box, which has a detection limit of <0.010 μM $NO_x$ (15). $NH_4^+$ was measured using the orthopthaldialdehyde fluorescence method (16). The detection limit for $NH_4^+$ is 0.030 μM. The sum of $NO_x$ and $NH_4^+$ is reported as dissolved inorganic nitrogen (DIN). Dissolved inorganic phosphorus as orthophosphate (DIP) was measured using the colorimetric method, with a detection limit of 0.030 μM (17).

**Enzyme analysis.** The substrate 4-methylumbelliferyl phosphate (MUF-P; Sigma-Aldrich) was used to assay AP activity, and L-leucine-4-methyl-7-coumarinylamide hydrochloride (Leu-AMC; Sigma-Aldrich) was used to assay LAP activity. Briefly, ~20 $cm^3$ of rock (weighed at the end of the experiment) was placed into a 125-ml Nalgene bottle, followed by the addition of 30 ml of refrigerated (4°C) buffer (Trisma pre-set crystals, 0.05 M, pH 8.5; Sigma-Aldrich) (18). The buffer pH was within the midrange of pH optima of AP enzymes reported in the literature (19), as well as the pH optima reported for LAP (20). For sample J2482, the amount of rock sample was limiting, so only LAP was assayed. Two additional bottles per experiment were run as negative controls to test for the abiotic breakdown and autofluorescence of the substrate over time. These bottles received the Trisma buffer but no sample and were treated like the experimental samples.

Selected volumes of substrate were added to each container of rock and buffer to produce a range of substrate concentrations that covered a broad range of enzyme activities (21) so as to facilitate modeling of $K_m$ and community maximum potential enzyme activity ($V_{max}$). For Leu-AMC, the substrate concentrations used were 4.1, 8.2, 10.3, 20.5, and 41.1 μM. For MUF-P, the substrate concentrations used were 1.67, 3.33, 6.94, and 13.9 μM. After the addition of the substrate, incubations were maintained in sealed 125-ml Nalgene bottles at 4°C, the *in situ* temperature for the sampling site. At 0 h, 1 h, and 3 to 4 additional time points over a 25-h period, overlying water samples were pipetted into 2-ml microcentrifuge tubes and immediately frozen. At the end of each experiment, the incubated rocks were stored in sterile centrifuge tubes at −80°C until they were analyzed at the University of Southern California.

Fluorescence was measured using a Turner Designs fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. The overlying water samples in microcentrifuge tubes were centrifuged for 7 min at 14,000 × *g* using a desktop nonrefrigerated ultracentrifuge, during which time the samples defrosted and particles were removed (18). After centrifugation, 1.3 ml of overlying water was removed and added to 1.3 ml of 0.5 M (pH 10.3) buffer ($NaCO_3$-$NaHCO_3$). The differences between the pH 8.5 buffer used during the experiment and the pH 10.3 buffer used during fluorescence quantification permitted the assay to be run at an environmentally appropriate pH and the MUF-P and Leu-AMC activities to be read at the optimum pH for the maximum fluorescence of their fluorophores. MUF-P and Leu-AMC were calibrated against standards made using stock solutions of 100 mM fluorophore dissolved in methanol. Buffer was used for subsequent dilutions. The substrate hydrolysis rates were calculated by linear regression of the increase in fluorophore concentration over time. These rates were used to determine the $V_{max}$ through nonlinear least-squares fitting of the Michaelis-Menten equation.

**Adsorption test on rocks using standard substrates.** A separate experiment was done using selected substrate concentrations of fluorophores to measure any possible adsorption of these substrates onto basalt rocks. This experiment was done using the same rock chips from the live experiment, which were defrosted, kept at 4°C, and washed in cold sterile buffer prior to the adsorption experiment. Triplicate bottles were initiated for three concentrations of standard (AMC and MUF) with no associated substrate (phosphate or leucine) added. Fluorescence was measured immediately and several more times over a 24-h period.

**Comparison of extracellular enzyme data from other sites.** In order to compare the enzyme activities measured here to those reported previously from diverse environments, it was necessary to convert units. Data from previous studies of extracellular enzyme activities in the water column are generally presented as nmol liter$^{-1}$ h$^{-1}$. To convert to nmol g$^{-1}$ h$^{-1}$, we divided the values by 1,000 (1 ml ≈ 1 g). To convert sedimentary data, reported as nmol cm$^{-1}$ h$^{-1}$, we multiplied the values by 1.5 (22). To convert data from mangrove sediments, reported as nmol (g dry sediment)$^{-1}$ h$^{-1}$ (23), we applied a correction factor to adjust to nmol (g wet sediment)$^{-1}$ h$^{-1}$ by multiplying values by 0.15 (24).

**DNA extraction and processing for biomass and community structure analysis.** A cetyltrimethylammonium bromide (CTAB) phenol-chloroform extraction was used (25) to extract DNA from seafloor basalts. The three initial samples and three endpoint (24-h time point) samples from LAP assays with an 8.2 M Leu-AMC substrate concentration were analyzed. qPCR was used to estimate the prokaryotic abundance. qPCR results for sedimentary prokaryotes are precise and estimates of the percentages of *Bacteria* among total prokaryotes are accurate, but actual estimated cell numbers are potentially inaccurate (26). Therefore, we view the qPCR results as estimates of prokaryotic biomass that allow the determination of cell-specific activity. qPCR for bacteria was carried out as described previously (27) using primers 338f (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 518r (5′-ATT ACC GCG GCT GCT GG-3′) and assuming 3.9 16S rRNA gene copies per cell. qPCR for archaea was carried out using primers 806f (5′-ATT AGA TAC CCS BGT AGT-3′) (28) and 922r (5′-YCC GGC GTT GAN TCC AAT T-3′) (29) and assuming 1.8 16S rRNA gene copies per cell (27). The thermal program employed for both bacterial and archaeal qPCR primer sets was as follows: 10 min at 95°C followed by 45 cycles of 30 s at 95°C, 30 s at 55°C, and 25 s at 72°C. The melt curves for all qPCR products were checked to ensure that a single PCR product was generated. qPCRs were run in triplicate.

PCR of the V6 hypervariable region of the 16S rRNA gene was carried out as described previously (30). PCR amplicons were cleaned using Zymo DNA Clean and Concentrator-5 spin columns, and then Ion Torrent sequencing adapters and barcodes were annealed to the cleaned PCR products. PCR amplicons from each sample were barcoded and combined into a single multiplex library, which was subsequently processed for sequencing using the Ion Torrent OneTouch machine (31) and 200-bp chemistry. An Ion Torrent 314 chip was used for sequencing.

The phylogenetic affiliations of the tag sequences (here referred to as pyrotags) were identified using the Global Alignment for Sequence Taxonomy (GAST) method (32) for all samples. Diversity statistics were calculated in Mothur (33) on samples trimmed down to an equal number of tags through random resampling. All statistics were calculated using the preclustering option in Mothur, which preclusters at a 2% difference level (1-bp difference for the V6 tags used here) using modified single linkage (34).

**Data processing for Ion Torrent-sequenced pyrotags.** The sample-processing pipeline developed for analyzing pyrotag libraries sequenced with the Ion Torrent consists of removing all sequences of >130 bp and then removing all sequences of <90 bp. Next, sequences with a mean quality score of <25 are removed. Then, forward and reverse primers are removed, with no mismatches allowed: a 100% match to both one of the five forward and one of the four reverse V6 primers (35) is required. The RDP website (36) is used for this purpose. Finally, the GAST program, available at http://vamps.mbl.edu/resources/software.php, was used to assign taxonomy. For V6 pyrotags designated "unknown" by GAST, each individual sequence is checked with BLASTN to determine whether an assignment can be made. RDP is used for this purpose, with the confidence cutoff set to 80%. If a sequence is not assigned to the domain *Bacteria*, it is removed from the data set. We note that while users of Roche 454 sequencers often remove any sequence with an N in it from their data sets (because the presence of an N indicates an overall low quality for that sequence), Ion Torrent does not assign the base N, so this is not possible.

**Screening of microbial genomes for the potential to produce LAP and AP.** To determine which of the top 20 bacterial orders recovered were potentially capable of extracellular enzyme production, we searched the Integrated Microbial Genomes (IMG) database (37) for genomes within these orders using the Find Functions search and the Enzymes (list) filters. EC 3.4.11.1 was used to search for the gene(s) encoding LAP, and EC 3.1.3.1 was used to search for the gene(s) encoding AP. Genomes were scored as positive if they contained a gene allowing the production of LAP or AP, and then the number of genes within the order was tallied to calculate the percentage of genomes within that order capable of producing each enzyme. This analysis depends on a few assumptions, as follows: (a) if an organism is a member of a clade in which a specific enzyme is more abundant, then that organism is more likely to have the enzyme, (b) the annotations in IMG are reliable, and (c) the AP or LAP is expressed extracellularly rather than intracellularly. However, in regard to the first assumption, while horizontal gene transfer of extracellular enzymes is possible, it was recently found that the genetic potential to produce AP shows a significant nonrandom association with phylogeny (38); therefore, it is more likely than not that the ability to produce extracellular enzymes follows phylogeny.

**Nucleotide sequence accession numbers.** Raw sequence files from this project can be obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive under project accession number SRP041681 and run accession numbers SRR1271392, SRR1271467, SRR1271468 to SRR1271470, and SRR1271472.

## RESULTS

**Microbial community biomass.** The prokaryotic abundances and community compositions of the samples from the three sites were estimated for the initial and final time points. The estimated total prokaryotic biomass from all sites ranged from $9.1 \times 10^5$ to $5.6 \times 10^6$ cells g$^{-1}$ rock, of which bacteria represented 91 to 99% (Table 1). At the end of the experiment, the prokaryotic biomass had increased slightly in both J2481 and J2482 (no J2477 sample remained to test).

**Activity potential of extracellular enzymes.** Potential LAP and potential AP activities were fitted to a Michaelis-Menten kinetics parameter (Fig. 2). Both the standard error calculations and the goodness of fit of curves (Table 2) gave confidence to the 1- to 2-h time point ($t_1$) estimates presented here. The extracellular enzyme activities and $V_{max}$ values were higher on basalt J2482 than on rocks J2481 and J2477 for both LAP and AP [$V_{max}$ values of 0.90 and 60 nmol (g rock)$^{-1}$ h$^{-1}$, respectively] (Table 2). Comparisons of the latter two site samples for LAP did not reveal a detectable difference between them. The $V_{max}$ was highest for both enzymes at $t_1$ and decreased with increasing incubation time. The $t_1$ results had the best fit to the model and were therefore selected for comparison of enzyme kinetics between samples (Table 2 and Fig. 2). The $K_m$ values for LAP at $t_1$ ranged from 26 to 33 μM and did not show the dramatic difference between stations that was seen for $V_{max}$ values. The $K_m$ values for AP were higher for station J2477 (7 μM at $t_1$) than for J2482 (2 μM at $t_1$). The activities of all enzymes measured were considerably higher than those of blanks run on buffer samples alone, so that the subtraction of blank values did not affect the potential expression of activity;

**TABLE 1** Prokaryotic abundances[a] and overlying water nutrient chemistry

| Sample[b] | Bacterial cells $g^{-1}$ | Archaeal cells $g^{-1}$ | Total prokaryotic cells $g^{-1}$ | % *Bacteria* | DIN (μM) | DIP (μM) |
|---|---|---|---|---|---|---|
| J2481 | $2.97 \times 10^6 \pm 8.2 \times 10^5$ | $6.81 \times 10^4 \pm 1.9 \times 10^3$ | $3.04 \times 10^6$ | 97.8 | ND[c] | ND |
| 481end | $5.45 \times 10^6 \pm 1.2 \times 10^6$ | $1.38 \times 10^5 \pm 1.9 \times 10^4$ | $5.59 \times 10^6$ | 97.5 | | |
| J2482 | $8.35 \times 10^5 \pm 1.3 \times 10^5$ | $6.97 \times 10^4 \pm 1.3 \times 10^3$ | $9.05 \times 10^5$ | 92.3 | 44.32 | 2.76 |
| 482end | $2.53 \times 10^6 \pm 1.1 \times 10^6$ | $2.42 \times 10^5 \pm 1.6 \times 10^4$ | $2.77 \times 10^6$ | 91.3 | | |
| J2477 | ND | ND | ND | ND | 36.84 | 2.57 |
| 477end | $3.21 \times 10^6 \pm 3.0 \times 10^5$ | $4.13 \times 10^4 \pm 3.2 \times 10^3$ | $3.25 \times 10^6$ | 98.8 | | |

[a] Prokaryotic abundances were estimated using qPCR.
[b] Samples are from initial (J2477, J2482, and J2481) and end (477end, 482end, and 481end) time points.
[c] ND, not determined.

the activities in the LAP blank at $t_1$ were 0.0084 nmol $liter^{-1}$ $h^{-1}$ at 10 μM substrate added and 0.0161 nmol $liter^{-1}$ $h^{-1}$ at 40 μM substrate added. For AP, the activities in the blank at $t_1$ were 0.79 nmol $liter^{-1}$ $h^{-1}$ at 7 μM substrate added and 1.85 nmol $liter^{-1}$ $h^{-1}$ for 27 μM substrate added.

**Adsorption of enzyme substrate on rocks.** The potential for adsorption of enzyme substrates on rocks was tested as a possible experimental artifact that might cause an apparent decrease in the potential enzyme activity as a function of time. The raw fluorescence graphed versus the standard concentrations added indi-

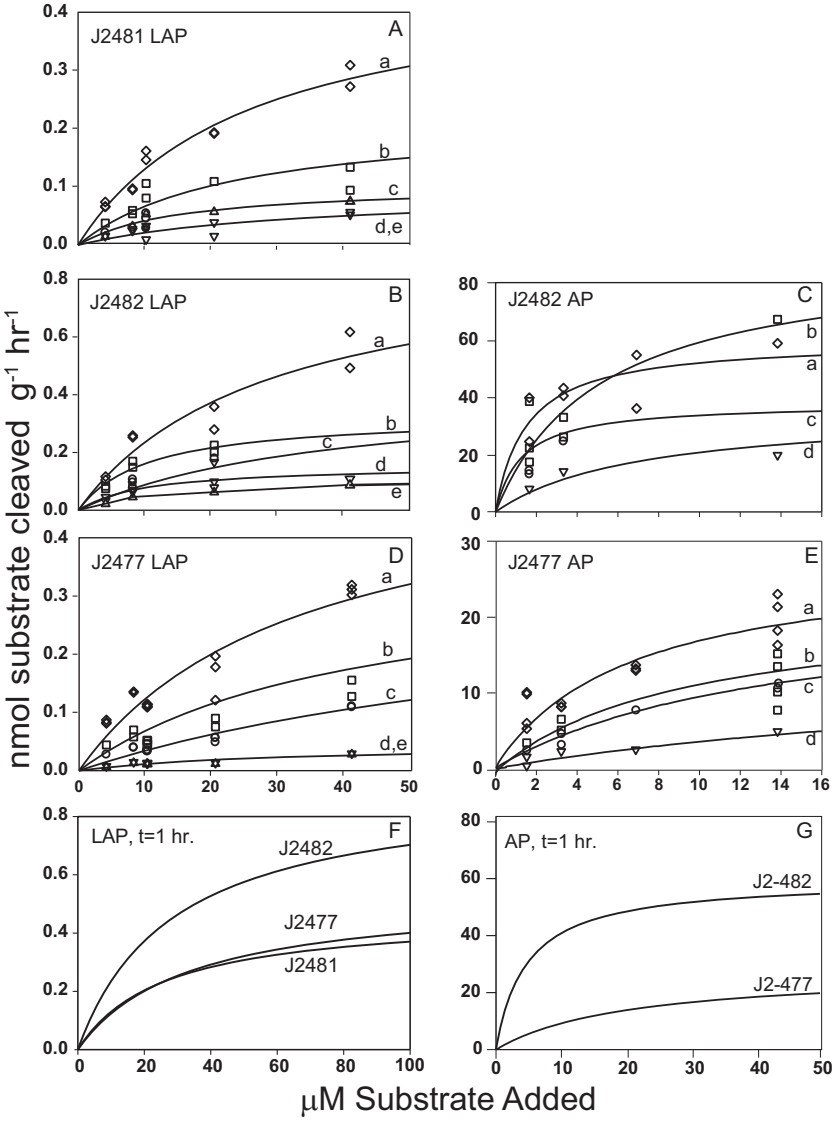


**FIG 2** Extracellular enzyme activity rates measured for LAP (A, B, D) and AP (C, E) on basalts from Loihi Seamount. The letters a to e indicate the five sampling time points per incubation. Diamonds represent time point $t_1$, squares $t_2$, circles $t_3$, upside-down triangles $t_4$, and right-side-up triangles $t_5$. A Michaelis-Menten kinetics model was fit for each time point; panels F and G compare models at $t_1$ for LAP (F) and AP (G).

**TABLE 2** Michaelis-Menten kinetics parameters calculated from raw enzyme data

| Enzyme, sample, and time (h) | Time point | Curve fit[a] | $K_m$ | SE ($K_m$) | $V_{max}$ | SE ($V_{max}$) | $R^2$ |
|---|---|---|---|---|---|---|---|
| Leucine aminopeptidase | | | | | | | |
| J2477 | | | | | | | |
| 1.5 | $t_1$ | a | 33.1 | 12.2 | 0.53 | 0.11 | 0.86 |
| 3 | $t_2$ | c | 33.2 | 15.7 | 0.25 | 0.67 | 0.25 |
| 6 | $t_3$ | b | 85.9 | 54.8 | 0.33 | 0.16 | 0.33 |
| 25 | $t_4$, $t_5$ | d,e | 29.5 | 26.0 | 0.04 | 0.02 | 0.88 |
| J2481 | | | | | | | |
| 1.5 | $t_1$ | a | 25.9 | 5.38 | 0.47 | 0.52 | 0.98 |
| 3 | $t_2$ | b | 8.77 | 4.29 | 0.14 | 0.03 | 0.71 |
| 7 | $t_3$ | c | 18.5 | 60.2 | 0.11 | 0.22 | 0.33 |
| 13 | $t_4$ | d | 33.4 | 29.0 | 0.09 | 0.45 | 0.61 |
| 25 | $t_5$ | e | 14.9 | 6.83 | 0.01 | 0.02 | 0.90 |
| J2482 | | | | | | | |
| 2 | $t_1$ | a | 28.5 | 14.3 | 0.90 | 0.24 | 0.94 |
| 4 | $t_2$ | c | 10.4 | 3.73 | 0.33 | 0.51 | 0.96 |
| 8.5 | $t_3$ | b | 37.9 | 13.0 | 0.42 | 0.98 | 0.98 |
| 13 | $t_4$ | d | 10.3 | 8.20 | 0.16 | 0.48 | 0.77 |
| 27 | $t_5$ | e | 15.7 | 4.70 | 0.12 | 0.15 | 0.98 |
| Alkaline phosphatase | | | | | | | |
| J2477 | | | | | | | |
| 1.5 | $t_1$ | a | 6.60 | 2.20 | 28.0 | 4.2 | 0.82 |
| 3 | $t_2$ | b | 14.6 | 16.9 | 22.7 | 15.0 | 0.86 |
| 6 | $t_3$ | c | 13.8 | 5.10 | 22.6 | 4.80 | 0.97 |
| 26 | $t_4$ | d | 30.1 | 41.6 | 14.4 | 14.5 | 0.95 |
| J2482 | | | | | | | |
| 2 | $t_1$ | a | 1.5 | 0.88 | 59.7 | 9.43 | 0.75 |
| 4 | $t_2$ | b | 5.1 | 2.79 | 89.5 | 24.3 | 0.87 |
| 8.5 | $t_3$ | c | 1.3 | 1.51 | 38.0 | 10.8 | 0.55 |
| 27 | $t_4$ | d | 7.0 | 4.41 | 35.3 | 7.70 | 0.95 |

[a] Letters refer to the labeled lines in Fig. 2.

cated no loss of fluorescence over 24 h, the length of the live experiments (Fig. 3). This is interpreted as no adsorption of the standards onto the rocks within the time and concentrations that were tested.

**Microbial community structure.** *Alpha-*, *Delta-*, and *Gammaproteobacteria*, *Bacteroidetes*, and *Planctomycetes* were the dominant bacterial groups present on the basalts sampled (Fig. 4). *Zetaproteobacteria* and *Firmicutes* were notably more abundant on basalt J2477 than on J2481 and J2482, while *Chloroflexi* and *Deferribacteres* were present on J2482 but nearly absent on the other two samples. There was a shift in community composition by the final sampling point, referred to here as $t_{end}$ (the 24-h-time-point samples from LAP assays with an 8.2 μM Leu-AMC substrate concentration), such that all of the $t_{end}$ bacterial communities were statistically more similar to each other than to their time zero counterparts. The proportions of *Alphaproteobacteria* decreased for all three samples during the incubation, but no other changes were consistent across all three samples. Rarefaction analysis revealed that the $t_{end}$ samples from all three sites were more diverse than the original samples (Fig. 5), which likely drove the observed similarity between the $t_{end}$ samples.

The 20 most abundant bacterial orders strongly reflected the most abundant phyla, mentioned above (Fig. 6). The orders *Acidimicrobiales* (phylum *Actinobacteria*), *Deferribacterales* (phylum *Deferribacteres*), unclassified taxa in the class *Holophagae* (phylum *Acidobacteria*), *Campylobacterales* (class *Epsilonproteobacteria*), and *Mariprofundales* (class *Zetaproteobacteria*) were also among the top 20 bacterial orders. *Xanthomonadales* was the most commonly detected bacterial order.

## DISCUSSION

In this study, both enzymatic and DNA-based approaches were used to characterize the potential activity and diversity of microbial communities on seafloor-exposed basalts. The work presented here suggests that extracellular enzymes can be used as metabolic proxies, in conjunction with other measures, to address hypotheses related to mechanisms of microbial survival and nutrient cycling rates in basalt-hosted environments.

**Enzymes from basalt-hosted microbial communities.** These are the first measurements of active microbial metabolism on seafloor basalts. In comparison on a per-volume basis with enzyme activities measured in other marine environments (Fig. 7), the LAP activity on Loihi basalts is lower than that measured on marine snow particles (39), in a coral reef mound (40), and in mangrove sediments (23). The basalt LAP activity rates overlap the lower end of sedimentary activities (5, 41), are at the higher end of rates from the water column in fjords (42), and are higher than data from the marine water column (6, 43). The AP activity rates measured on Loihi basalts are within the midrange of activities measured in marine sediments (44), seawater particles (39), a cored coral reef mound (40), and subsurface hydrothermal fluids (45). The basalt AP activity is higher than water column measurements (43, 46).

Fewer reports are available on cell-specific enzyme activity

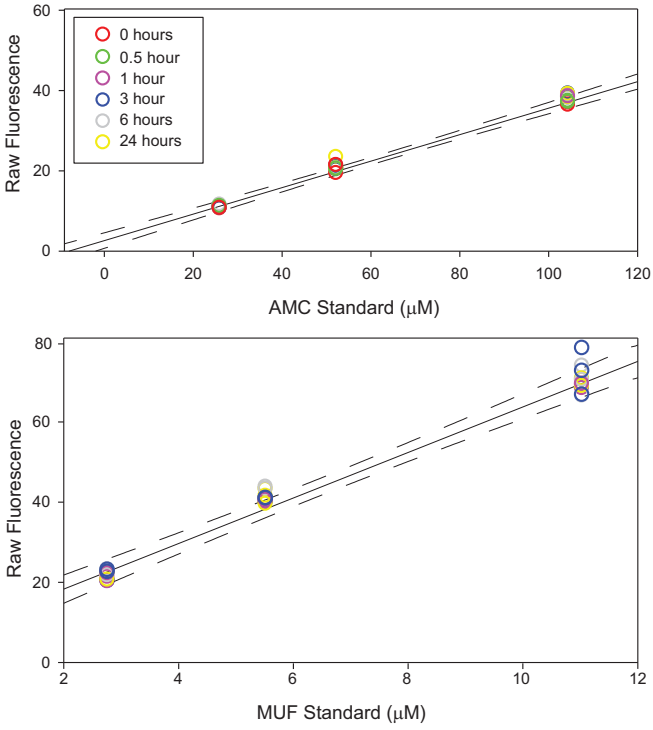


**FIG 3** Raw fluorescence versus concentration of standard added to bottles in adsorption experiment.

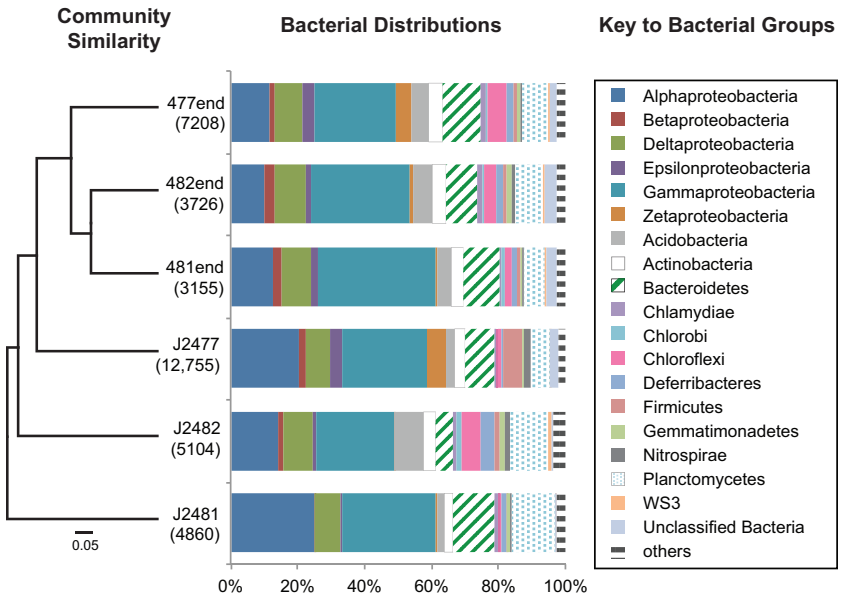


**FIG 4** Bacterial distributions in initial samples (J2477, J2482, and J2481) and at final time points (477end, 482end, and 481end). The end samples analyzed were from the 8.2 μM substrate concentration assays for LAP. Bacterial phyla/classes are shown for those groups representing >1% of the total community in at least one sample. Dendrogram at left represents community similarity between samples measured using Bray-Curtis and Yue-Clayton (with complete agreement between the methods) at the 97% similarity level. Numbers in parentheses are total number of V6 sequences per sample. For the community similarity analysis, all samples were randomly resampled down to 3,155 sequences.

than on community activity, and therefore, we focused our comparison on seawater, particle, and sediment cell-specific LAP activities because the most data exist for these environments. The cell-specific LAP activity in basalts falls on the higher end of the ranges measured for seawater and particles/aggregates in seawater (Fig. 7) (43, 47–49). The cell-specific LAP activities in marine sediments have the highest measured values (5, 41).

AP production generally seems to be regulated by a need for inorganic phosphate, as well as the presence of organic phosphate. Heterotrophic organisms may also use AP to relieve secondary limitation of carbon or nitrogen should the organic P molecule contain such moieties (9, 10, 12, 44, 50), but it is currently unclear whether AP production is also stimulated by nutrient limitation related to carbon limitation or whether this is an opportunistic utilization of constitutive enzymes present in microbial cells. There is experimental support for increased turnover rates of P

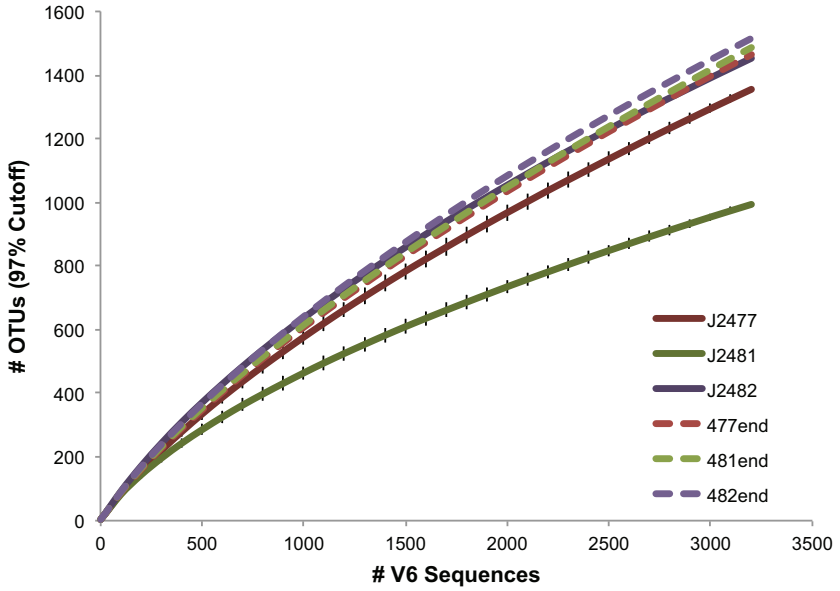


**FIG 5** Rarefaction analysis of V6 sequences in initial samples (J2477, J2482, and J2481) and at final time points (477end, 482end, and 481end). Vertical lines for J2477 and J2481 are 95% confidence limits, which show that these two samples were statistically different than the others. The other four samples were statistically the same.

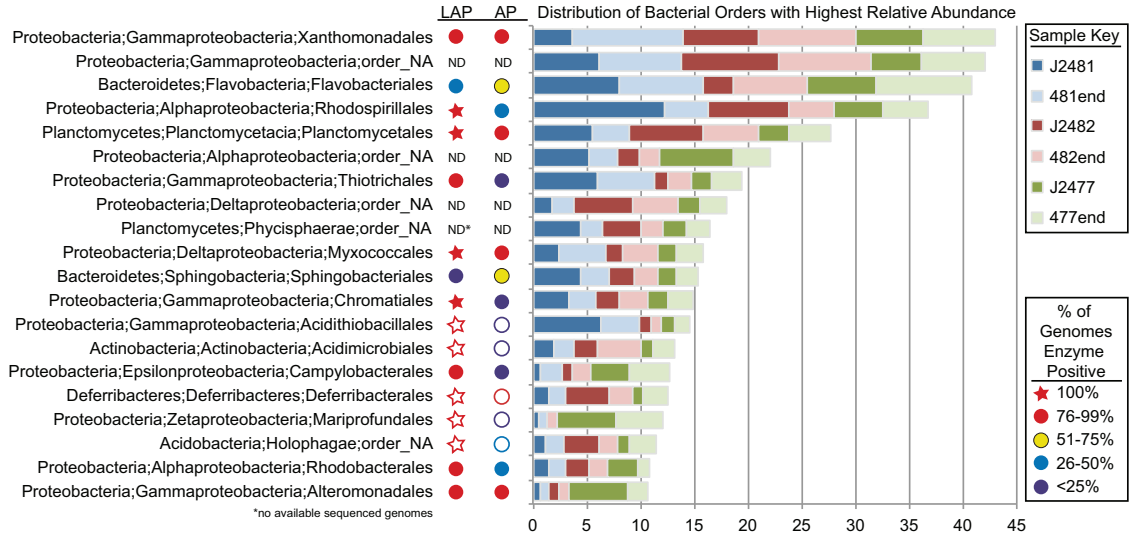


**FIG 6** Distribution of the top 20 bacterial orders recovered from the initial and final time points. The percentages of sequenced genomes containing LAP and AP are indicated. Closed symbols indicate orders for which >10 sequenced genomes are available at the Integrated Microbial Genomes website (37). Open symbols indicate that <10 genomes are available. The *x* axis measures the relative abundance of taxa.

species in response to C limitation, indicating a role for AP in obtaining organic C (9, 51–53). There is also strong support in the literature for the organic and inorganic P concentration being inversely proportional to AP activity (6, 54). It is likely that the broad substrate specificity of AP indicated by the results just discussed implies that AP enzymes contribute to the processing of organic P moieties using different regulation systems in different environments. The nutrient chemistry of background water from the sites sampled here (Table 1) indicates that inorganic N and P are likely not in limited supply, and therefore, the enzyme activities at Loihi might be functioning to liberate organic C for microbial consumption.

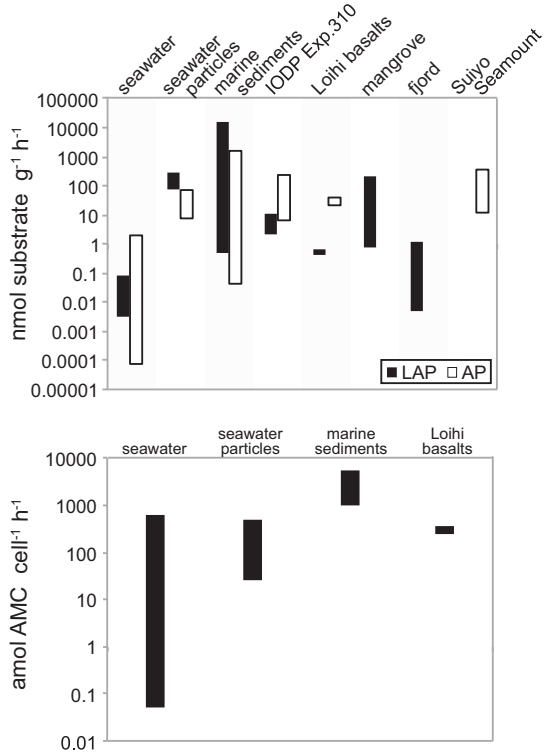


**FIG 7** Comparison of enzyme activities from this study and previous studies. Top, data include LAP and AP activities from various substrates normalized to nmol $g^{-1}$ $h^{-1}$. Data used derive from this study and original data and summary tables in references 5, 6, 23, 39, 40, 42 to 46, and 68. Bottom, data include LAP activities normalized to amol $cell^{-1}$ $h^{-1}$. Normalized cell data derive from references 5, 41, 43, and 47 to 49.

LAP hydrolyzes leucine from the N terminus of polypeptides and has been shown experimentally to be responsive to the addition of amino acids (55). However, there is no simple relationship between bulk measures of N availability and microbial N-acquiring LAP activity (6). Since amino acids and amino sugars can be sources of C as well as N, it is difficult to assign one discrete function of this enzyme directly in deep-sea environments. It is possible that LAP can be thought of as an indicator of overall microbial activity (56). In support of this rationale, the respiration rates in grassland soil are directly related to LAP activities (57). However, it is clear that since there is a cost of enzyme upregulation and production, the activity ratios of this enzyme indicate a response to some signal of need or stress, whether it be nitrogen or carbon based or due to another stimulus.

The ratio of LAP activity to AP activity has been used as an indicator of relative nutrient limitation in a number of studies. Williams et al. (58) found that this ratio approaches 1:1 with increased levels of dissolved organic C and dissolved organic N. In our study, the LAP/AP ratios were 1:50 at J2482 and 1:20 at J2477. Greater AP activity in this study can be interpreted as a higher demand for phosphorus and/or carbon. However, as mentioned above, inorganic N and P are likely not limiting in this environment, and therefore, the observed AP activity, as well as the LAP/AP ratio, is likely be more related to organic C acquisition (10) than to a need for DIP.

The loss of extracellular enzyme activity with time seen here (Fig. 2 and Table 2) has also been described in other studies (5, 44). An experiment to quantify this loss did not indicate any measure-

able loss related to adsorption of the fluorophores tested. It is possible that the substrates rather than the fluorophores are responsible for adsorption phenomena observed, perhaps with binding to iron oxide or other metal moieties. This may be exacerbated by the fact that in the live experiments, the microbial cells responsible for the observed activity are likely attached to the incubating rocks. The fluorescent product liberated from the substrate may also stick to the rocks and so not be detected by sampling the water column. If this happens at a constant rate, then less substrate is available over time and the observed activity will decrease because less product will be produced. The decrease of $V_{max}$ with time may also be ascribed to a decrease in enzyme activity due to lysis of cells or changes in species composition and/or biomass during the experiment. We are unable to point to an obvious reason for the decreasing enzyme activity with time in this experiment; the microbial biomass and bacterial species composition were not indicative of drastic changes. It is possible that changes in substrate availability might be the best predictors of the observed decrease in activity. It is also possible that the enzyme activity indicates changes in the microbial community that, due to the short time course of this experiment, were not detectible in the quantity of biomass and bacterial species composition. Previous work illustrated that LAP and AP remain active over a 36-h incubation time (59), and therefore, we believe the presentation of extracellular enzyme activity kinetics for our 1-h time period is reflective of *in situ* activities.

Michaelis-Menten kinetics is used in this study to describe and compare enzyme activities across sampling areas and time. $K_m$ in any environment is related to reductions in substrate conversions (6), which are controlled by substrate affinity to the enzyme (which may be affected by pH or temperature). Substrate availability is also controlled by production and diffusion, as well as competitive and noncompetitive inhibition. $K_m$ values therefore reflect environmental effects on enzyme regulation, as well as individual cell-specific enzyme levels (60). The actual concentration of enzyme available is reflected in the magnitude of $V_{max}$. Therefore, a low $V_{max}$ is an indication of a lower enzyme concentration, which might be interpreted as a reflection of numbers of organisms, the historical production of enzyme found in an environment, and the general metabolic activity of the area (i.e., less enzyme indicates fewer active microbes given the same limiting conditions). A pattern of low $V_{max}$ and higher $K_m$ was seen in AP activities, where J2482 had a higher $V_{max}$ and lower $K_m$ than J2477, indicating higher enzyme activity or affinity at the first site and, potentially, more enzyme at the second site. With LAP, although $V_{max}$ differed between sites, there was no significant difference in $K_m$. These results imply that, for LAP, there is no difference in effective binding efficiencies between sites, while for AP, there may be. In addition, there may be inhibition of enzyme-substrate interactions by many mechanisms, including but not limited to selective adsorption and competitive inhibition.

**Microbial biomass and species characterization.** These are the first deep-sequencing results for seafloor basalts. Previous studies have illustrated that basalts are host to diverse and abundant microbial communities dominated by bacteria (2, 4, 27, 61–64). Pyrotag sequencing of the V6 hypervariable region of 16S rRNA supports earlier findings that *Gammaproteobacteria* are the most abundant bacterial class on seafloor basalts and that the *Chromatiales* and *Thiotrichales* orders of sulfur-oxidizing bacteria are among the basalt-associated *Gammaproteobacteria* (2, 4, 62). The deeper sequencing afforded by the Ion Torrent, a next-generation platform, allowed the detection of some bacterial orders that were not previously observed on seafloor basalts. The order *Acidithiobacillales* was found to be common on Loihi basalts, especially on basalt J2481 (Fig. 7). This order was not previously detected on basalts, perhaps due to the smaller numbers of sequences generated by sequencing full-length clones, as done in prior studies. The *Acidithiobacillales* were recently proposed to be a new class within the *Proteobacteria* (65), and seafloor basalt may be an ideal environment to study their physiology. The orders *Deferribacterales* and *Mariprofundales* were both also previously undetected on seafloor basalts.

Microbial lineages are not universally capable of extracellular enzyme production (38); therefore, we searched the IMG database (37) to determine whether the top 20 lineages in our samples are potentially capable of LAP or AP production. We found that, with the exception of the phylum *Bacteroidetes*, most of the bacteria represented in the 20 most abundant orders recovered are predicted to be capable of LAP production (Fig. 7). Therefore, it is impossible to predict which taxa are most likely responsible for the LAP activity observed. Conversely, the ability to produce AP was more variable, even within the same bacterial class. *Xanthomonadales*, *Flavobacteriales*, and *Planctomycetales* were all abundant on Loihi basalts and are also capable of AP production, indicating that these orders were potentially also responsible for much of the measured AP activity. With the data from this experiment, it is impossible to make any stronger connections between enzyme activities and the taxa responsible, but future work can employ targeted PCR and qPCR and/or omics-based approaches to tease apart these relationships.

**Contribution of basaltic rocks to biogeochemistry of the ocean.** Basalts that are exposed or thinly sedimented cover approximately 600,000 km$^2$ of the ocean floor (1). Using our measured enzyme activities, we have roughly estimated the contribution of basaltic rocks to the Earth's ocean activity. We assume, conservatively, that the top 1 cm of exposed basalts are in dynamic communication with seawater. We then calculate the total basalt enzyme activity relative to that of other global environments as follows:

$$V_{max,environment,global} = \nu_{environment} \times V_{max,specific\ environment}$$

where $V_{max,environment,global}$ is the global potential for potential enzyme activity in a given environment, $\nu_{environment}$ is the volume of the chosen environment, and $V_{max,specific\ environment}$ is the volume-specific $V_{max}$ for a particular enzyme in a particular environment. In basalts, $\nu_{environment}$ is given as the product of the area of exposed basalts (600,000 km$^2$) and the relevant depth (1 cm). The input parameters and results are presented in Table 3 and Fig. 7. All LAP measurement estimates cited are broad ranges that take into consideration the high and low estimates from the papers cited. In addition, there is uncertainty associated with the depth into basalts that are in active communication with seawater, as well as the variability of enzyme activities in basalts. Nevertheless, the results demonstrate that the potential for exposed basalts to transform organic matter is substantial; far greater than the total potential of the marine water column and comparable to the total potential of shelf sediments. We propose that basaltic rock plays a significant, quantifiable role in benthic biogeochemical processes. Future experimental work will more precisely quantify the calculations presented here.

**TABLE 3** Estimates of global LAP rates for selected marine environments

| Environment | Vol ($cm^3$)[a] | Total hydrolytic potential (LAP) (nmol $h^{-1}$)[b] |
|---|---|---|
| Basalts, seamounts, and knolls | $2 \times 10^{24}$ | $9 \times 10^{23}$ to $1.4 \times 10^{24}$ |
| Tropical mangroves | $1 \times 10^{10}$ | $0.14 \times 10^{6}$ to $38 \times 10^{6}$ |
| Slope sediments | $2 \times 10^{23}$ | $9.6 \times 10^{22}$ to $3 \times 10^{27}$ |
| Total water column above 200 m | $3 \times 10^{23}$ | $1.6 \times 10^{22}$ |
| Total water column below 200 m | $1 \times 10^{25}$ | $6 \times 10^{20}$ |

[a] Volumes of habitats are derived from data in references 23, 66, and 67.
[b] Activity measurements were calculated from data in references cited in the legend to Fig. 7.

**Summary and conclusions.** These are the first measurements of active microbial metabolism on seafloor basalts. The enzymatic activities measured here are orders of magnitude higher than those observed in the water column for both LAP and AP (Fig. 2) (6, 46). The LAP activities measured on Loihi basalts overlap the low end of those measured in marine sediments (5, 41), while the AP activities on Loihi basalts are within the middle of the range of those measured in sediments and on subsurface rocks at Suiyo Seamount, the only other site where AP has been measured for marine rocks (45). We determined the potential taxa involved in AP and LAP production and activities and found that the putative ability to produce LAP is evenly distributed across the most commonly detected bacterial orders but the ability to produce AP is likely dominated by bacteria in the orders *Xanthomonadales*, *Flavobacteriales*, and *Planctomycetales*. Our activity estimates indicate that this vast area of the ocean benthos has a great potential to contribute to ocean biogeochemistry, further quantification of which requires further study.

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